# DIVISION S-3—NOTES

## METHODOLOGICAL VARIABILITY IN MICROBIAL COMMUNITY LEVEL PHYSIOLOGICAL PROFILES

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### Abstract

**We performed two experiments to assess the methodological variability of microbial community-level physiological profiles (CLPP, using the BiOLOG assay) in soil from a California annual grassland ecosystem. In a study to assess the impact of sample preparation, we found that bacteria adhered to soil surfaces are numerically dominant and have CLPPs indistinguishable from that of intact soil. Studies that allow soil particles to settle prior to dilution or plating may not accurately reflect the substrate utilization pattern of whole soil. In a hierarchical ANOVA, we found that nearly all of the methodological variability in the CLPP assay comes from soil replicates rather than plate replicates. Many laboratories replicate at the level of the CLPP plate. Our results indicate that to best represent a given soil sample, it is important to replicate soil subsamples, rather than CLPP plates.**

The importance of microbial communities in soil functioning, such as nutrient cycling and carbon storage, has led to the development of methods for describing and quantifying microbial community characteristics including biomass, nitrogen content, activity, and measures of functional, taxonomic, and genetic diversity (Zak et al., 1994; Tiedje et al., 1999). Community-level physiological profiling of the microbial community (e.g., the BiOLOG assay) has been used to characterize microbial communities based on a pattern of substrate utilization in 96-well microtiter plates (Garland and Mills, 1991). While there is some criticism of the CLPP method because it is based on bacterial culturability (Konopka et al., 1998), it nevertheless continues to be used by research groups interested in functionally based microbial community assay. The CLPP method is inexpensive, fast, reliable, and easy to use. In addition, comparisons have shown that CLPP methods are comparable with other community assays in their ability to discriminate among a wide range of soil microbial communities (Haack et al., 1995; Garland, 1996; Kennedy and Gewin, 1997; Palojarvi et al., 1997; Carpenter-Boggs et al., 1998; Balser, 2000). However, there are aspects of CLPP methods that merit further study. In particular, optimal procedures for sample replication and handling have been the subject of recent debate (Hitzl et al., 1997; Mayr et al., 1999; Lowit et al., 2000).

The BiOLOG assay was originally developed as a way to characterize the functional ability of bacterial isolates. It has since been used widely for soil and water samples (Garland, 1996; Lowit et al., 2000). Whole soil presents several problems that are not encountered with isolates or water samples. First, soil must be diluted to reduce the particulate load prior to plating. Dilution reduces the number of bacterial cells per milliliter of solution, and may result in uneven distribution of rare bacteria from well to well. In addition, bacteria are not evenly distributed within soil; they tend to be clumped, adhering to particles. Many groups using the BiOLOG assay disperse the intact soil and allow it to settle prior to dilution or plating. This treatment allows characterization of only the cells in the soil solution and those that are easily removed from soil surfaces (see Wünsche et al., 1995; Staddon et al., 1997, 1998). The validity of this approach has been questioned; it is uncertain whether it accurately reflects the intact soil (Bossio and Scow, 1995; Mayr et al., 1999). We present results from a study that compares CLPPs from suspended soil, soil-solution-only, and soil particle-adhered communities.

In addition, we assess the information gained by replicating BiOLOG plates vs. soil sub-samples in a CLPP study. Many groups inoculate replicate plates from a single soil sample (Knight et al., 1997; Goodfriend, 1998; Lowit et al., 2000), yet the value of plate replicates vs. soil subsample replicates has not been thoroughly evaluated. In order to optimize effort and resources in using CLPP methods, it is useful to consider the sources of variability in the assay (Montagna, 1982). We performed a nested ANOVA to determine the sources of variability in the BiOLOG CLPP assay at the level of the soil replicate, the dilution series, and the inoculated plate.

### Materials and Methods

#### Field Site

Soil was sampled from a California annual grassland site in the foothills of the Southern Sierra Nevada mountains. The ecosystem is a blue oak annual grassland savanna, composed primarily of a blue oak (*Quercus douglasii* Hook. & Arn.) overstory (≈60% cover), and an annual grassland understory (Trumbore et al., 1996). The climate is Mediterranean, with rainfall concentrated from November to February. The soil is in the Fallbrook soil series (fine-loamy, mixed, thermic Typic haploxeralfs) and is formed on weathered granodiorite (Trumbore et al., 1996). The soil has a clay content of 10% (v/v), total C content of 1.9% (v/v), and a pH (1:1 soil:0.1 *M* CaCl) of 5.5. A surface organic horizon overlying the mineral soil persists throughout the year, ranging in depth from 1 to 2 cm.

#### Sampling and Laboratory Methods

We present results from two experiments. In the first experiment, we used hierarchical sampling in order to quantify the impact of soil dilution and to design a sampling scheme that

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**Abbreviations:** CLPP, community-level physiological profiles; PC1, first principal component; PC2, second principal component; PCA, principal components analysis.

minimizes the impact of primary sources of variability in the CLPP method. We used a nested ANOVA, beginning with a single composited sample from a grassland soil. We then subsampled, made a dilution series, and inoculated BiOLOG microtiter plates in triplicate. The result was 27 plates inoculated from a single soil sample.

The second experiment was designed to assess variability resulting from the treatment of soil prior to plating. We asked whether easily extractable bacteria differed functionally from those adhered to soil particles, and assessed the potential for biased subsampling of the soil community. Using a sequential extraction procedure to isolate easily extractable and adhered bacteria for analysis with CLPP, we determined whether bacteria sampled from the solution phase of a soil dilution accurately reflected the substrate utilization profiles of the intact community.

### Soil Preparation

For both experiments, the soil was homogenized by thorough mixing and removing coarse roots and fragments by hand. For the nested ANOVA experiment, we used three 5-g subsamples from a 500-g composited soil sample. Each 5-g subsample was suspended in 50 mL of 50 $\mu M$ phosphate buffer (pH 7.1). This initial 1:10 dilution was shaken vigorously for 30 min on a reciprocal shaker. Three replicate dilution series (to $10^{-3}$ dilution) were then prepared from each subsample, and three plates from each dilution series, to yield 27 plates inoculated from the original soil sample.

For the sequential extraction experiment, we added phosphate buffer (pH 7.1) in a 10:1 ratio to triplicate soil subsamples (≈25-g oven dry equivalent). Samples were shaken vigorously for 30 min. Soil particles were removed from solution by gently centrifuging ($1000 \times g$) the sample for 15 min, and decanting the supernatant to a clean sample container. The supernatant contained the *suspended* cell community. Cells in the pellet were the *particulate* microbial community.

We also investigated the use of a more vigorous extraction procedure, adding a surfactant (Triton X-100, Sigma-Aldrich, St. Louis, MO) during extraction to remove cells adhering weakly to soil surfaces (Bakken, 1985). We used the centrifuge and decant method described above to separate aqueous and particle communities. The result of this extraction was not numerically different in terms of cell numbers from the aqueous community, nor did it have a distinguishable CLPP. For the remainder of this paper, we discuss results from the initial aqueous and particulate community separation only.

### Inoculation of BiOLOG Plates

For our nested ANOVA, we plated the $10^{-3}$ dilution of each dilution series into triplicate BiOLOG plates, adding 150 μL per well with an 8-channel multipipetter. The $10^{-3}$ dilution had $\approx 1.5 \times 10^6$ cells $mL^{-1}$ (by acridine orange direct counts). In our sequential extraction experiment, we inoculated plates with one of three community types. We used the supernatant without further dilution (the *supernatant* community), we resuspended the soil pellet to a $10^{-3}$ dilution after centrifugation (the *particulate* community), and we used $10^{-3}$ dilutions of unmanipulated soil suspensions (*intact* soil community). Using this dilution scheme, the three community types had similar inoculum densities (Table 1). We ran three replicates of each community type.

All plates were incubated at 28°C, and read on a BiOLOG Microplate Reader (at 590-nm wavelength; BiOLOG Inc., Hayward, CA) approximately every 12 h. The plates were considered finished or fully developed when the average color development in the wells was between 0.75 and 1.0. This typically occurred between 36 and 48 h.

**Table 1. Cell counts and inoculum densities from the sequential soil extraction experiment.**

| Community type | Cell counts | Inoculum density |
|---|---|---|
| | million cells $g_{soil}^{-1}$ | thousand cells $mL^{-1}$† |
| Intact | 111 ± 23 | 11.1 |
| Particulate | 108 ± 10 | 10.8 |
| Suspended‡ | 0.63 ± 0.06 | 6.3 |

† Soil inoculum is based on the dilution factor for each community type. Intact soil was diluted to $10^{-3}$ g $mL^{-1}$, particulate samples were resuspended to a dilution of $10^{-3}$ g $mL^{-1}$, and suspended cells were used directly (cells removed from soil solids in a 1:10 extraction).

‡ Suspended cells are those remaining in solution following centrifugation to remove soil solids. Cell count is based on direct counts of supernatant samples, and is back-calculated to a per gram soil basis.

### Data Manipulation

We subtracted color development in the control well from absorbance readings in all other wells. Negative values were set to zero. We chose the time point to analyze for each plate based on its average well color development, as per Garland (1996). Prior to statistical analysis, we normalized individual well absorbance by total plate color to account for possible differences in inoculation density between samples. We used these processed data for factor analysis and other calculations to generate variables as described below.

### Acridine Orange Direct Counts

We quantified the bacterial numbers present in intact, aqueous, and particulate-only soil communities using epifluorescence microscopy. We made direct counts of the initial 1:10 dilution, as well as of the aqueous and particulate preparations using acridine orange stain. Nucleopore-black filters were stained for 5 min each, and were mounted on glass slides with paraffin oil under cover slips. We counted 48 fields per slide, and three slides per community sample. There were three soil replicates per aqueous, particle, and whole soil community type, for a total of nine slides counted per community type. All materials were prepared using glassware washed to remove cells, and filtered distilled water. We counted several prepared blank slides and subtracted the results from sample slide cell counts.

### Statistical Tests

Principal components analysis (PCA) is a mathematical technique that allows multivariate data to be characterized by a smaller number of variables. The first principal component (PC1) is the linear combination of the original variables that represents the greatest spread observed in the data, and can be used as a summary of the multivariate observations (Selvin, 1995). In contrast to other multivariate ordination techniques (such as discriminatory analysis, or correspondence analysis), PCA is unbiased by a priori assumptions about treatment effects. Because of the unbiased nature of the analysis, univariate statistical procedures such as plots, correlations, t-tests, and ANOVA techniques can be applied to the principal component values to describe relationships under study (Selvin, 1995). We used the PC1 from our CLPP data as a summary variable to perform a nested ANOVA. The statistical model was:

$$Y_{ijk} = \mu + T_i + D_{ij} + e_{ijk},$$

where $Y$ is the length of PC1 for each observation (a univariate representation of the entire CLPP), $\mu$ is the overall sample mean, $T_i$ is PC1 for each soil subsample (each tablespoon of soil from the original 500-g soil sample), $D_{ij}$ is PC1 for each

**Table 2. Nested analysis of variance results, using the first principal component (PC1) from all data as the summary variable. The first and second factors (PC1 and PC2) accounted for 58 and 14% of the variance in the data set, respectively.**

| Source | df | MS | *F* | *P* | Variance from given source |
|---|---|---|---|---|---|
| | | | | | % |
| Soil subsample | 2 | 4.899 | 56.25 | <0.005 | 78 |
| Dilution series | 6 | 0.0871 | 1.567 | <0.25 | 4.1 |
| Plate | 15 | 0.0556 | | | 17.9 |

dilution series, and $e_{ijk}$ is the random error due to PC1 from each replicate plate. The model for the variance ($\sigma^2$) was:

$$\sigma_y^2 = \sigma_T^2 + \sigma_D^2 + \sigma^2.$$

To compare aqueous, particulate, and intact soil subcommunities, we first performed PCA on the total data set, then used one way ANOVA followed by Tukey's HSD test to assess differences among the three communities. To visualize the microbial community profiles we plotted the PC1 and the second principal component (PC2) in a two-dimensional plot. We used JMP statistical software (SAS Inc., Carey, NC) for all analyses.

## Results and Discussion

### Nested Analysis of Variance

To address the issue of replication in CLPP assays, we quantified sources of methodological variance in soil with a nested ANOVA. As shown in Table 2, nearly all of the variability in analysis of three replicate soil samples could be accounted for at the level of the soil subsample. Replicate dilution series and plates were reproducible and highly homogeneous (accounting for only 4 and 18% of the overall variability, respectively). This result is in agreement with a CLPP study by Mills and Bouma (1997); however, it is in contrast to other microbiological studies and techniques. In a study using epifluorescent microscopy (acridine orange direct cell counts), Montagna et al. (1982) found that replicate slides of a single water sample were responsible for the majority of methodological variability. Using CLPP, Lowit et al. (2000) found that plate replicates were necessary to properly characterize river water samples. There are important differences in the CLPP method applied to soil vs. water samples: in contrast to soil samples, water samples are relatively homogeneous and well mixed. There is no need for initial dilution to remove opaque matter. Whole soil must be diluted prior to addition to a CLPP plate, and is highly heterogeneous (Mills and Bouma, 1997). Despite these differences between soil and aqueous samples, many research groups working with soil CLPP assays incubate replicate plates from a single soil sample. Our results indicate that replication at the plate level is of little value, and the effort would be better spent on additional soil subreplicates.

### Sequential Extraction

To compare the easily removed (suspended) and particle-adhered microbial communities, we used PCA to summarize the CLPP data and to visualize general differences in substrate utilization among suspended, particulate, and intact soil communities (Fig. 1). Intact and particulate communities were similar relative to the suspended community. The intact and particulate profiles both differed from that of the suspended community. The standard errors associated with PC1 and PC2 were much larger for the supernatant community-type than those for either the particle or intact communities (Fig. 1). In addition to differences in patterns of substrate utilization, there were also differences in the number of cells found in each of the extractable communities (Table 1). The particulate community accounted for a large percentage of the cells. The difference in cell counts between suspended and particulate communities is not unexpected. It has long been recognized that the most cells in the soil are attached to surfaces, and are not easily removed from the soil (Stotsky, 1986). While it is possible that low cell density could be the cause of greater variability in the CLPP profile, in the case of our data we do not think this is the cause. First, we inoculated the CLPP plates with the supernatant directly (no additional dilution), and diluted the particle samples in order to have a consistent number of cells $mL^{-1}$ inoculant (Table 1). Second, for the PCA we used data from the time point for each plate having similar color development. There were no significant differences among the community types in overall response, nor number of positive responses.

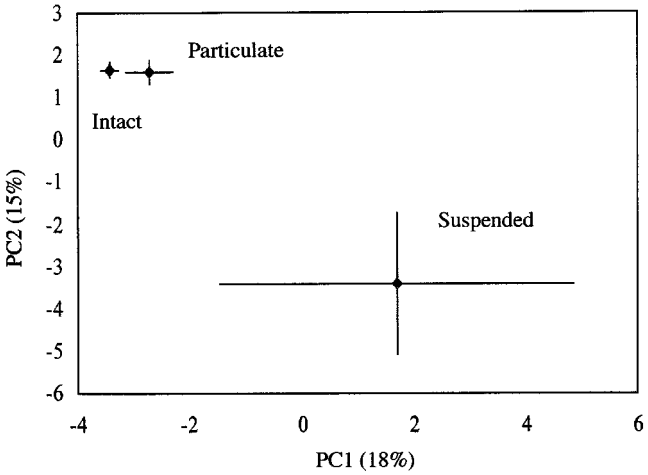


**Fig. 1. Ordinate plot from principal components analysis on BiOLOG data from the grassland site, showing the profile from intact soil compared with that from the particulate and supernatant microbial communities in the soil. The ANOVA with the first principal component (PC1) as the dependent variable and community type as independent has an $R^2 = 0.413$ ($P < 0.025$). The community types do not separate out significantly along the second principal component (PC2) by community type.**

While our results are based on only one soil type, they nevertheless point toward two characteristics of soil bacterial communities: (i) particulate communities may comprise the numerically dominant portion of the intact soil community; and (ii) the substrate utilization profile of intact soil communities (plated from a soil slurry) may not be well represented by the suspended bacterial community (plated after allowing soil to settle). Soils of differing texture will settle at different

rates, or the soil disruption procedure used (sonication, shaking, or blending) may have different dispersal efficiencies, resulting in methodological variance and inconsistent results. We found that removing soil solids prior to plating can alter CLPP results substantially. Other studies have also shown that bacteria in repeated extractions of the same soil differ in composition and activity (Bååth, 1996; Kreitz and Anderson, 1997; Mayr et al., 1999).

## Conclusions

Our findings have implications for optimal utilization of the CLPP assay. Many research groups currently using CLPP allow the soil in their dilutions to settle prior to plating (Wünsche et al., 1995; Knight et al., 1997; Staddon et al., 1997, 1998). The results from this experiment indicate that the aqueous-extractable community comprises a relatively small subset of the intact community, and can differ substantially in carbon utilization from the whole-soil microbial community. The variability of CLPPs from particle-adhered bacteria was much lower than that for the suspended community profiles. Differences in settling time may influence results independent of real soil differences, and studies that allow soil to settle prior to plating may not accurately represent the soil community. We recommend performing dilution series by shaking each sample, and immediately transferring an aliquot of the soil slurry. This should minimize particle settling, and increase the accuracy of the assay.

In order to streamline a method, a simple nested ANOVA can provide information about methodological variability. A soil CLPP assay is the most variable at the level of the soil subsample, rather than at the level of the plate. This is in contrast to studies using CLPP on aqueous samples. Replication at the level of plates or dilution series is an inefficient use of time and money. We recommend reducing sample variance by increasing the size or number of soil subsamples and incubating one plate per sample.

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# DIVISION S-4—SOIL FERTILITY & PLANT NUTRITION

## Contributions of Shoot and Root Nitrogen-15 Labeled Legume Nitrogen Sources to a Sequence of Three Cereal Crops

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### ABSTRACT

**Legume mulches are important sources of N for cereal crop production, particularly for organic and resource-poor producers. A field study was conducted using a direct method to determine if the amount of N in cereal crops derived from either the shoots or roots of preceding tropical legume cover crops was affected by their chemical composition and mineralization potential. *Desmodium ovalifolium* Guill. & Perr. [= *D. adscendens* (Sw.) DC. and *Pueraria phaseoloides* (Roxb.) Benth.], were grown in 6.0-m$^2$ microplots and foliar-labeled with 99 atom % $^{15}$N urea. A cereal sequence of maize (*Zea mays* L.)–rice (*Oryza sativa* L.)–maize followed the legumes. Cereal accumulation of legume N from either the shoot (shoot + leaf litter) or the root-soil sources was evaluated by spatially separating the legume N sources. This was achieved by interchanging surface applications of nonlabeled and $^{15}$N-labeled legume shoots with in situ $^{15}$N-labeled and nonlabeled legume roots. Initially the *Desmodium* shoot N source contained 316 kg N ha$^{-1}$ and roots contained 12.5 kg N ha$^{-1}$. *Pueraria* shoots and root N sources initially contained 262 and 14.8 kg N ha$^{-1}$, respectively. About 90 g kg$^{-1}$ of the initial N of each legume shoot was recovered in the total aboveground tissues from the three cereal crops, while 490 g kg$^{-1}$ of *Desmodium* and 280 g kg$^{-1}$ of *Pueraria* root-soil N sources were recovered. Of the 181 kg N ha$^{-1}$ accumulated aboveground by the cereal sequence, the contribution of shoot plus root-soil N sources was 200 g kg$^{-1}$ from *Desmodium* and 150 g kg$^{-1}$ from *Pueraria*. Cereal N was derived primarily from mineralization of soil organic matter present before the legumes and possibly from N deposition (precipitation and dry) occurring during the cereal crop sequence. After harvest of the last cereal crop, 13 and 180 g kg$^{-1}$ of the initial legume N was present as inorganic and organic N fractions, respectively, in the top 75 cm of soil. Even though *Pueraria* shoots had a lower C:N ratio and concentration of polyphenols than *Desmodium* shoots, the relative contributions of the shoot N source were similar for both legumes. Decomposition of legume residues, particularly legume shoots, make a meaningful contribution to the N economy of cereal crops grown in the tropics. The legume cover crops (root + shoot) contributed nearly 280 g kg$^{-1}$ of the aboveground N in the first cereal crop and as much as 110 g kg$^{-1}$ of the N in the third crop during the 15-mo sequence of cereals.**

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Legumes are used commonly in agricultural systems as a source of N for subsequent crops and for maintaining soil N levels. This use is particularly important in the humid tropics where N fertilizers often are not economically feasible due to poor market and infrastructure development (Palm and Sanchez, 1991). To date, studies attempting to quantify the legume N contribution to subsequent crops have been conducted mainly in temperate agroecosystems and have dealt primarily with aboveground legume N, ignoring root N because of the difficulty of harvesting roots and nodules. Moreover, these assessments of N cycling in cover crop based production systems have often relied on indirect methods that evaluate plant and soil N pools (Ditsch et al., 1993; Luna-Orea and Wagger, 1996), N release from cover crop residue (Ranells and Wagger, 1991; Luna-Orea et al., 1996), and N uptake by a summer crop (Hargrove, 1986; Clark et al., 1994).

Nitrogen-15 methodology is useful for resolving N dynamics, whereby $^{15}$N-labeled legume cover crops are harvested and applied as N sources for subsequent grain crops (Varco et al., 1989; Jordan et al., 1993; Harris et al., 1994). Varco et al. (1993) found that 600 g kg$^{-1}$ of the N was mineralized and subsequently lost from $^{15}$N-labeled hairy vetch (*Vicia villosa* Roth) residue 30 d after surface application, yet an average of only 60 g kg$^{-1}$ was recovered as soil inorganic N for two growing seasons. In Australia, Ladd and associates (Ladd et al., 1981, 1983; Ladd and Amato, 1986) reported field-grown wheat (*Triticum aestivum* L.) recovered between 11 and 280 g kg$^{-1}$ N from $^{15}$N-labeled medic (*Medicago littoralis* L.) and an additional 40 g kg$^{-1}$ recovery by a second wheat crop. When $^{15}$N-labeled red clover (*Trifolium pratense* L.) residue was applied at maize planting, 150 g kg$^{-1}$ was recovered in the harvested crop and 570 g kg$^{-1}$ was retained by the soil (Harris et al., 1994). Of these studies, that of Varco et al. (1989) made an indirect estimate of the contribution of legume root N to subsequent crops. Only a few direct estimates of N contribution from legume shoot and root residues are available (Harris and Hesterman, 1990; Russell and Fillery, 1996).

The objectives of this study were to: (i) quantify the N contribution from two tropical legume cover crops of differing chemical characteristics (i.e., potentially dif-